Estrogen treatment enhances hereditary renal tumor development in Eker rats

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Hormonal influences are known to affect the development of renal cell carcinoma in man and laboratory animal models. We tested the hypothesis that estrogen treatment or ovariectomy of rats modulates renal tumor development using tuberous sclerosis 2 (Tsc2) heterozygous mutant (Eker) rats in which a germline mutation predisposes the animals to renal cell tumor development. Two-month-old female wild-type and Eker rats were ovariectomized or sham-operated and treated with placebo or 5 mg 17β-estradiol in s.c. pellets for 6 or 10 months. Rats were examined at 8 or 12 months of age, at which time the numbers of renal tumors and preneoplastic foci were quantitated and the severity of nephropathy was assessed. In contrast to what may have been expected, prolonged estrogen treatment enhanced the development of hereditary renal cell tumors, with a 2-fold greater number of preneoplastic and neoplastic renal lesions compared with untreated Eker rats. Ovariectomized Eker rats had 33% fewer renal lesions than the unmanipulated control group. No tumors or preneoplastic lesions were present in wild-type rats at either time point. Estrogen treatment increased the severity of nephropathy in both wild-type and Eker rats, whereas ovariectomy was protective against nephropathic changes. Although estrogen is not a rat renal carcinogen, it enhanced the development of hereditary renal cell tumors when administered to Eker rats. Eker rats heterozygous for a mutation in the Tsc2 locus provide a good model in which to study how genetic and hormonal factors contribute to the development of renal cell tumors and to understand the influence genetic susceptibility has on the development of renal cell carcinoma.

Introduction

Differences in incidence of renal cell carcinoma (RCC) that exist between males and females in both humans and laboratory animal models are probably due to hormonal influences. Male rats more frequently develop spontaneous and chemically induced renal epithelial tumors than female rats (1) and men spontaneously develop renal carcinoma twice as often as women (2–6). The findings of steroid hormone receptors in normal kidney and RCC tissue (5) in conjunction with the development of RCC in women following chronic estrogen therapy suggests that endocrine involvement is important in tumor development. Estrogen is known to be important in the development of several experimental tumors. For example, estrogen administration inhibits hepatocarcinogenesis in mice whereas ovariectomized mice have an increased neoplastic response (7).

In some tissues, estrogen may act to inhibit tumor progression, whereas in other tissues it appears to act as a tumor promoter. Estrogen acts as a tumor promoter in experimental rat liver carcinogenesis (7,8) and in spontaneous human endometrial (9,10) and mammary carcinomas (9,11). The role of estrogen in renal cell carcinogenesis is not well understood. Some renal cancers in human patients have been found to have endocrine dependency and are positive for estrogen receptors (12). Although chronic estrogen treatment induces kidney tumors in male hamsters, estrogen does not induce renal proliferative lesions in rats (8,13).

A rat model of hereditary RCC first described by Eker (14) was shown to be due to a germline mutation in the tuberous sclerosis 2 gene (Tsc2) (15,16). Tsc2 is a tumor suppressor gene and is thought to be specifically involved in the pathogenesis of renal cell carcinoma in humans and rats (17). In addition to renal cell tumors, characteristic preneoplastic dysplastic lesions have been described (18,19). The mutation of Tsc2 in the Eker rat is an insertion of 5 kbp of DNA of unknown origin into the 3′-portion of the Tsc2 gene that leads to a new 3′-terminus and elimination of the distal portion of the Tsc2 gene (20,21).

The present study was conducted to determine the role of estrogen in spontaneous rat renal tumorigenesis by examining the ability of 17β-estradiol and ovariectomy to modify the onset of hereditary renal cell tumors and their development in Eker rats. Since renal tumors are more common and numerous in males, it was hypothesized that estrogen treatment would inhibit and ovariectomy enhance hereditary renal tumor development. Female heterozygous Tsc2 mutant (Eker) rats were utilized since they are a well-characterized and sensitive model in which to examine factors that may influence renal cancer development.

Materials and methods

Female Tsc2 (Eker) mutant and wild-type rats on a Long-Evans genetic background were sham-operated or ovariectomized and treated with placebo or 17β-estradiol s.c. pellets. Unbalanced treatment groups were present in the experiment because the mutant status of individual rats was unknown at the outset of the experiment and a population-based design was used (22). At the completion of the in-life phase of the study, a published description of the molecular changes responsible for the Eker mutation allowed a PCR-based analysis to identify the Tsc2 status of individual animals (15,16; Table I).

A total of 180 female Long-Evans rats from a mutant Tsc2 carrier colony were randomly assigned to treatment groups by weight, 60 per treatment group. The rats were group housed two per plastic shoe box cage with direct

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contact cellulose bedding and uniquely identified by transponders and cage cards. The rats were kept on 12 h light–dark cycles with a room temperature of 20 ± 2°C and humidity of 50 ± 10%. Water and feed (NIH-07 chow; Zeigler, Gardener, PA) were available ad libitum. At 55–60 days of age, 60 rats were ovariecietomized under isoflurane anesthesia with the remaining 120 rats sham-operated on for gonadectomy. While under anesthesia, the rats had a 5 mg 17β-estradiol or placebo 90-day slow release pellet (Innovative Research of America, Toledo, OH) implanted s.c. between the dorsal scapulae.

Table I. Distribution of Tsc2 mutant and wild-type animals in each group

<table>
<thead>
<tr>
<th>Treatment</th>
<th>All animals</th>
<th>Carrier animals</th>
<th>Non-carrier animals</th>
<th>Unscheduled deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 months</td>
<td>12 months</td>
<td>8 months</td>
<td>12 months</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>18</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>30</td>
<td>13</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>Estrogen</td>
<td>30</td>
<td>20</td>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

The atypical hyperplasias have similar cell types but more variability in cell characteristics that included clear, basophilic and eosinophilic cell types. Non-neoplastic and neoplastic renal lesions are detailed and illustrated elsewhere (18). The morphological characteristics of the neoplastic and preneoplastic renal tumors and preneoplastic foci were identified based on standard criteria (18). The morphological characteristics of the neoplastic and preneoplastic renal lesions were determined by measuring the concentration of DNA by using the QIAamp tissue kit and following the method in Eker rats. Marked uterine atrophy was present in ovariectomized rats but no reproductive tract masses were noted.

Ovariectomy resulted in a trend toward increased body weight and average weight gain during the study, however, estrogen treatment resulted in a significantly lower body weight and average weight gain. Although there were treatment-related changes in weights, the numbers of preneoplastic foci and total lesions, treatment did not affect the incidence of development of uterine smooth muscle tumors or splenic vascular tumors, which are also associated with the Tsc2 mutation in rats. Marked uterine atrophy was present in ovariecietomized rats but no reproductive tract masses were noted.

Results

Treatment-related clinical observations were limited to weight loss and lethargy associated with developing pituitary masses in estrogen-treated rats. Of 60 rats treated with estrogen, 12 were removed early from the study due to morbidity from effects related to pituitary masses. Estrogen treatment did not affect the incidence of development of uterine smooth muscle tumors or splenic vascular tumors, which are also associated with the Tsc2 mutation in rats. Marked uterine atrophy was present in ovariecietomized rats but no reproductive tract masses were noted.

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Table II. Mean body weight and average gain of female Eker rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean body weight (8 months (g))</th>
<th>Average gain (8 months (g))</th>
<th>Mean body weight (12 months (g))</th>
<th>Average gain (12 months (g))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>286 ± 22</td>
<td>128 ± 21</td>
<td>319 ± 40</td>
<td>163 ± 39</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>297 ± 42</td>
<td>143 ± 42</td>
<td>350 ± 46</td>
<td>196 ± 45</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>242 ± 31</td>
<td>87 ± 27</td>
<td>214 ± 28</td>
<td>59 ± 28</td>
</tr>
</tbody>
</table>

*Significant decrease in body weight, P < 0.01.

The Eker mutation is defined by a 6.3 kbp insertion into the Tsc2 gene and this permits genotyping by PCR using specific primers for identification of the Eker (+/+)-mutant. Two primers designed to amplify the mutated Tsc2 mutant and wild-type animals in each group. The PCR conditions were used were 93°C for 30 s, 56°C for 30 s and 72°C for 1 min for 35 cycles. Aliquots of the PCR products were size separated on 1% agarose gels. Since the Eker homozygous mutant dies in utero, the possible allotypes of the viable progeny are either +/+ or +/+. Thus the individual animals need to be screened only for the presence or absence of the mutant 892 bp allele. Statistical analysis included ANOVA and the F-test for equal means, as well as the non-parametric Mann–Whitney U-test. By significantly different we mean that the hypothesis of equal means is rejected at a significance level of <0.01, in favor of the hypothesis that the treatment is different from the control.
Ovariectomy 13 110 \textsuperscript{a} (9 17) with the \textit{Tsc2} mutation predisposing them to RCT development. In addition to the effects on tumor development, estrogen increased and ovariectomy decreased the severity of nephropathy, suggesting a link between nephrotoxicity and tumorigenesis in this animal model.

Estrogen inhibits or stimulates tumor development in an organ- and cell-specific manner. Estrogen-enhanced tumor induction is proposed to be through non-genotoxic mechanisms of altered growth (24–28). Estrogen has been shown to inhibit the development of transplanted mouse mammary carcinomas (29) and diethylnitrosamine (DEN)-initiated mouse hepatocarcinogenesis (30) and may be the reason that female mice tend to have fewer spontaneous liver tumors than males (31). Chronic estrogen treatment is associated with greatly increased male rat mammary tumor incidence (13).

Estrogen-induced carcinogenicity has been linked to metabolism of estrogen to reactive catechols or quinones; however, these metabolites have limited affinity for the estrogen receptor (32). Catechol estrogen–glutathione conjugates cause oxidative damage and induce mild nephrotoxicity in the hamster model of estrogen-induced nephrocarcinogenicity. Catechol estrogens readily undergo oxidation to form orthoquinones which undergo redox cycling resulting in oxidative stress and can react directly with cellular nucleophiles such as protein, non-protein sulfhydrils and DNA (27,33). Kidney cells have been shown to have enzyme systems that metabolize estrogen to form catechol estrogens. The nephrotoxicity of polyphenolic–glutathione conjugates in rats is a consequence of the relatively high activity of \textit{γ}-glutamyltranspeptidase within the brush border membrane of renal proximal tubular epithelial cells (32). With chronic administration there is greater availability of estrogen metabolites with resultant formation of potentially damaging reactive species and enhanced oxidative stress in the kidney (34). Lipid hydroperoxide and protein carbonyl levels were substantially elevated in hamster kidneys after a single injection of \textit{17β}-estradiol that also produced mild toxicity in the proximal tubule epithelium (33,35). Chronic treatment with \textit{17β}-estradiol enhanced the development of nephrotoxicity in the present and previous studies in direct association with an increase in tumor multiplicity or incidence (24,25). The decreased nephrotoxicity in the present study was also associated with a marked decrease in tumor multiplicity.

Polyphenolic–glutathione conjugates are formed as metabolites of a variety of non-genotoxic carcinogens in rats, including the male rat nephrocarcinogen hydroquinone (36). Recently a potent nephrotoxic metabolite of hydroquinone, 2,3,5-(tris-glutathion-S-yl)hydroquinone, was shown to promote tumorigenesis in Eker rats (37). The induction of tumorigenesis in \textit{Tsc2} mutant rats in the present study is most probably associated with estrogen metabolism and nephrotoxicity.

Estrogen-induced renal carcinoma has been extensively studied in Syrian golden hamsters and much information is available concerning the metabolism of estrogen in this species (24,25,38). Less information is available concerning estrogen metabolic pathways, nephrotoxicity and nephrocarcinogenicity.

### Table III. Total number of neoplastic and preneoplastic lesions in one section of each kidney from female Eker rats at 8 months of age (mean ± SD)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of carriers</th>
<th>Atypical tubules</th>
<th>Atypical hyperplasia</th>
<th>Adenoma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>467 (26 ± 8)</td>
<td>82 (4.6 ± 2.4)</td>
<td>21 (1.1 ± 1.5)</td>
<td>1</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>13</td>
<td>110* (9 ± 3)</td>
<td>9° (0.7 ± 1.0)</td>
<td>14 (1.1 ± 1.0)</td>
<td>2</td>
</tr>
<tr>
<td>\textit{17β}-Estradiol</td>
<td>20</td>
<td>564 (28 ± 13)</td>
<td>147 (7.4 ± 6.5)</td>
<td>78 (3.9 ± 3.9)</td>
<td>4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Significant decrease in lesion compared with control, \(P < 0.01\).

\textsuperscript{b}Significant increase in lesion compared with control, \(P < 0.01\).

### Table IV. Total number of neoplastic and preneoplastic lesions in one section of each kidney from female Eker rats at 12 months of age (mean ± SD)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of carriers</th>
<th>Atypical tubules</th>
<th>Atypical hyperplasia</th>
<th>Adenoma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>362 (30 ± 8)</td>
<td>20 (2.1 ± 1.7)</td>
<td>11 (1.8 ± 1.8)</td>
<td>2</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>13</td>
<td>252* (19 ± 7)</td>
<td>14 (1.5 ± 1.1)</td>
<td>9 (1.2 ± 1.1)</td>
<td>4</td>
</tr>
<tr>
<td>\textit{17β}-Estradiol</td>
<td>10</td>
<td>336 (35 ± 16)</td>
<td>119° (14.6 ± 5.8)</td>
<td>55° (11.9 ± 7.9)</td>
<td>5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Significant decrease in lesion compared with control, \(P < 0.01\).

### Table V. Mean nephropathy scores for female \textit{Tsc2} mutant (Eker) rats at 8 and 12 months of age and wild-type rats at 12 months of age

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carrier rats 8 months of age</th>
<th>Carrier rats 12 months of age</th>
<th>Wild-type rats 12 months of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.7 (18)</td>
<td>1.3 (12)</td>
<td>0.8 (14)</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>0.2 (13)</td>
<td>0.8 (13)</td>
<td>0.4 (14)</td>
</tr>
<tr>
<td>\textit{17β}-Estradiol</td>
<td>2.3 (20)</td>
<td>3.1 (10)</td>
<td>2.8 (8)</td>
</tr>
</tbody>
</table>

Discussion

The present study showed that estrogen promoted and ovariectomy inhibited renal cell tumor (RCT) development in rats with a mutation predisposing them to RCT development. In addition to the effects on tumor development, estrogen increased and ovariectomy decreased the severity of nephropathy, suggesting a link between nephrotoxicity and tumorigenesis in this animal model.
in rats, a species less sensitive to estrogen-modulated renal tumorigenesis. Estrogen did not enhance dimethylnitrosamine (DMN)-induced kidney tubular adenomas and carcinomas nor did estrogen alone cause rat renal tumors (13). In the present study, although renal tumor development was enhanced in Tsc2 mutant rats, estrogen did not induce renal tumors in wild-type rats.

Estrogen-treated rats in the present study had a decreased body weight that was maintained throughout the study, but ovariectomized rats had increased body weight compared with sham-operated placebo-treated controls. In previous studies, chronic estrogen treatment in rats caused growth retardation, which was seen as poor weight gain (39,40). This is thought to be due to decreased feed conversion in spite of greater food consumption (39). In the present study, chronic estrogen treatment caused severe nephrotoxicity and subsequent regeneration of renal tubules. In contrast, ovariectomy prevented nephropathy. These data are in contrast to reports where nephrosis was less severe in rats treated with a combination oral contraceptive compared with controls (40). The marked severity of nephropathy in the present study was unexpected, since female rats typically have less severe nephropathy than males. High estrogen doses may be metabolized differently than physiological levels, resulting in nephrotic biochemical changes (24,25).

Although the results of the present study indicate a strong association between estrogen-induced nephrotoxicity after chronic high doses and renal tumorigenesis, hormonal influences cannot be completely ruled out. Hormonal modulation has been reported to affect RCC development. An increased risk of RCC was reported in multiparous women who have had five or more children and women who had a hysterectomy and an oophorectomy. In addition, women who have used oral contraceptives have a decreased risk of developing RCC (5). Some renal cancers in human patients have been shown to have estrogen receptors and previous work with estrogen-induced renal cancer in hamsters indicate that antiestrogenic compounds might have therapeutic benefit. However, the use of the antiestrogenic cancer chemotherapeutic tamoxifen in renal cancer patients failed to enhance recovery (12,41,42). The rat kidney possesses a receptor that responds to 17β-estradiol (43). Therefore, the tumor promoting effect of estrogen in the present study might have been partially mediated through the estrogen receptor. Estrogens may also up-regulate autocrine or paracrine polypeptide growth factor signaling pathways whose effects may be mediated by the estrogen receptor (44–47). Polypeptide growth factors, such as transforming growth factor α (TGFα), may modulate the effects of steroid hormone receptors (44). Overexpression of TGFα has been consistently reported in association with the development of renal neoplasms due to estrogen administration to hamsters (48). Eker rat renal tumors overexpress the polypeptide growth factor TGFα (49). Constitutive overexpression of TGFα in Eker rat renal tumors may have acted in concert with estrogen to promote the development of renal tumors (49). In the absence of physiological estrogen due to ovariectomy, tumor development was inhibited. When estrogen was removed by ovariectomy, it may no longer be available to interact with TGFα, resulting in inhibition of tumor growth.

Testosterone causes renal hypertrophy of the tubules, with estrogen producing the opposite effect. In the present study, neither estrogen treatment nor ovariectomy significantly affected kidney weight and estrogen appeared to stimulate a proliferative response in the tubules. The proliferative response may have been secondary to the nephrotoxicity and it also stimulated tumor development (43,50).

The specific mechanisms by which estrogen enhanced and ovariectomy inhibited hereditary renal tumor development were not determined. Persistent cytotoxicity with cell regeneration associated with nephrotoxicity may have stimulated estrogen-induced tumorigenesis (38,51). In addition, estrogens can induce cell replication by stimulating the synthesis of mitogenic growth factors and their receptors (51,52). The present study suggests that female Tsc2 mutant rats are a useful animal model for mechanistic studies of estrogen-induced nephrotoxicity and nephrocarcinogenicity.

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References


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